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The *Saccharomyces cerevisiae NPS1* gene, a novel *CDC* gene which encodes a 160 kDa nuclear protein involved in G_2 phase control

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We have cloned the gene *NPS1* (nuclear protein of *Saccharomyces*) which encodes a nuclear protein of mol. wt 156 735 Daltons (1359 amino acids) essential for cell growth. *NPS1* contains a 2 kb sequence that is highly homologous to the *S.cerevisiae SNF2/GAM1* gene known as a transcriptional regulator for multiple genes. However, the *NPS1* gene was found to have a distinct function from *SNF2/GAM1*. The growth of the cells carrying a *nps1Δ::URA3* deletion allele and galactose-inducible *NPS1* on a plasmid was arrested under *NPS1*-repressed conditions with a cell cycle arrest phenotype, being arrested at the large-bud stage with a single nucleus that had a DNA content of G_2/M phase. When the arrested cells were further incubated under *NPS1*-repressed conditions, re-replication of DNA occurred in some of the arrested cells without passage through mitosis. In the predicted amino acid sequence of *NPS1*, sequences homologous to the catalytic domain of protein kinases were found. We constructed a mutation which results in the substitution of a highly conserved lysine residue (Lys792) in the presumed ATP-binding site of this kinase-like domain with a glutamic acid codon. The mutant gene failed to rescue the growth defect caused by *NPS1* disruption, suggesting that Lys792 is essential for the function of *NPS1*.

Key words: CDC gene/G_2 phase control/nuclear protein/protein kinase/*S.cerevisiae*

Introduction

Every eukaryotic cell proliferates through a strictly regulated process called the cell cycle. In the budding yeast, *Saccharomyces cerevisiae*, the genes involved in cell cycle regulation have been genetically studied using temperature sensitive lethal mutants (*cdc* mutants) whose growth is arrested at specific points of the cell cycle at the restrictive temperature. To date, >70 *cdc* mutants have been isolated and the outline of this regulatory network deduced (Pringle *et al.*, 1981). However, it is thought that more *CDC* genes remain to be identified.

Recently, we have cloned the gene *NPS1* which is essential for mitotic growth of the yeast *S.cerevisiae*. We show here that the gene is involved in G_2 phase control. In addition, we show genetic evidence that the *NPS1* product may have protein kinase activity.

Results

Cloning and DNA sequence determination of NPS1

NPS1 (nuclear protein of *Saccharomyces*) was cloned by chance from a yeast genomic library constructed in λgt11 in the course of our screening for the gene of a 70 kDa yeast nuclear protein p70 (detailed data for this 70 kDa protein will be described elsewhere). An isolated clone, KUT2, contained a 4.9 kb insert of yeast chromosomal DNA fragment in an *Eco*RI site of λgt11 (Figure 1). DNA sequence determination from the left hand *Eco*RI to *Bgl*II of KUT2 revealed the presence of a non-interrupted open reading frame (ORF) in this region. Search for identical sequences to DNA data bases indicated that this DNA fragment contained a novel sequence. To analyze the *NPS1* transcript, a probe derived from the 1.7 kb *Eco*RV−*Kpn*I fragment was hybridized to total RNA prepared from *S.cerevisiae* A364A cells grown to early-log, mid-log and stationary phases. As shown in Figure 2, the *NPS1* probe hybridized to the 4.8 kb mRNA. The level of *NPS1* transcription in stationary phase cells was notably lower than that in early and mid-log cells (Figure 2). This result suggested a possible involvement of *NPS1* in cell growth.

Because the λKUT2 insert lacked the 5′ end of the *NPS1* gene, we screened a yeast (A364A) genomic library

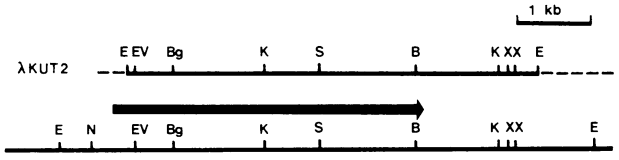

Fig. 1. Restriction map of the *NPS1* gene. Dashed and solid lines indicate λgt11 vector and yeast genomic DNA respectively. The arrow indicates the ORF of the *NPS1* gene deduced from its DNA sequence. Only selected restriction sites are shown. Abbreviations for restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; K, *Kpn*I; N, *Nhe*I; S, *Sac*I; X, *Xba*I.

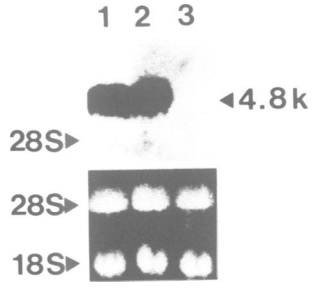

Fig. 2. Northern blot analysis of *NPS1* mRNA. Total RNA (20 μg) from A364A cells grown to early-log (lane 1), mid-log (lane 2) and stationary (lane 3) phases were prepared for hybridization with the 1.7 kb *Eco*RV−*Kpn*I fragment of *NPS1* labeled with [α-^{32}P]dCTP. The upper and lower panels show autoradiographic and ethidium bromide staining patterns of rRNA respectively.

constructed in the shuttle vector YIp1 (Suzuki *et al.*, 1983) for a full-length clone with a probe generated from the *Eco*RI−*Kpn*I fragment of KUT2. The isolated clone was named pKUT5. A restriction map of the genomic region surrounding the *NPS1* locus is shown in Figure 1. The complete sequence of 5211 bp DNA from the *Eco*RI site of pKUT5 was determined (the *NPS1* DNA sequence will appear in the EMBL, DDBJ and GenBank nucleotide sequence data bases under the accession number D01087). This gene encodes a 4077 bp ORF corresponding to a polypeptide of 1359 amino acids (156 735 Daltons, Figure 4), if the 5′-most methionine codon in the ORF is the initiation codon. We performed S1 nuclease mapping on poly(A)$^+$ RNA with a ^{32}P-labeled single-stranded probe complementary to the sequence between positions −709 and 273. Radiolabeled polynucleotides of 340 and 344 bp were protected from nuclease digestion (Figure 3, lane 1b, data for sequencing gel are not shown), suggesting that NPS1 mRNA is transcribed from guanine at −67 or from cytosine at −71, and methionine codon at position 1 is the translational initiation site.

At position 747, *NPS1* contains a TACTAAC box, a conserved sequence for intron splicing of yeast nuclear mRNA (Langford *et al.*, 1984). However, the entire sequence between *Pst*I and *Hin*dIII (positions 80 and 1171 respectively) used as a probe was protected from S1 nuclease digestion by hybridization with poly(A)$^+$ RNA (Figure 3, lane 3b). Thus, it was concluded that *NPS1* contained no intron.

The predicted NPS1 protein is rich in charged amino acids: the sum of aspartic acid and glutamic acid, and of lysine, arginine and histidine are 17 and 18% of the total residues

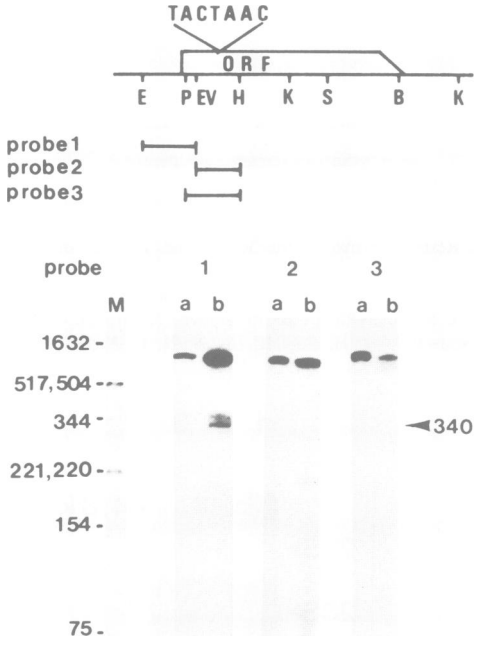

Fig. 3. Analysis of *NPS1* mRNA by S1 nuclease protection. The single-strand-specific probes complementary to the putative *NPS1* mRNA were used for hybridization. Positions and sizes of the probes are schematically indicated with the restriction map of the *NPS1* gene in the upper part of the figure. Abbreviations of restriction enzymes are as in Figure 1. Poly(A)$^+$ RNA was hybridized with the ^{32}P-labeled probe DNAs as described in Materials and methods. Probes and samples were run in lanes a and b respectively. Markers shown in bases on the left side are pBR322 DNA cut with *Hin*fI.

respectively. In the C-terminal region between the Arg residue at 1050 and the Lys residue at 1247, alternate clusters of basic and acidic amino acids are present. An amino acid sequence known as the recognition sequence motif of p34$^{cdc2/CDC28}$ kinase (Ser-Pro-Xaa-Lys/Arg, Shenoy *et al.*, 1989) is found at position 1231.

The *NPS1* DNA sequence was searched for identity to other sequences in DNA data bases. No sequence homology to *NPS1* was found in the data bases, but *NPS1* was found to contain a 2 kb sequence highly homologous to the *S.cerevisiae SNF2/GAM1* gene (Laurent *et al.*, 1991; Yoshimoto *et al.*, 1991) within its ORF. Nucleic acid sequence between positions 1309 and 3306 of *NPS1* shows 67% identity with that of *SNF2/GAM1* extending from positions 2271 to 4184. The predicted amino acid sequences of both genes within these regions also show high similarity. In Figure 4, we show the comparison of *NPS1* and *SNF2/GAM1* amino acid sequences aligned with the maximum matching program of DNASIS. Both sequences share nine highly homologous domains separated by eight less homologous ones (Figure 4). Within these nine domains, 97% similarity is found beteen the two genes if conservative amino acid substitutions are taken into consideration, with 83% identity. However, the remaining N- and C-terminal parts of each gene product are distinct from each other.

The *NPS1* protein (Nps1) sequence between residues 737 and 987 contains conserved sequence motifs characteristic of protein kinase catalytic domains. Hanks *et al.* identified 11 conserved subdomains shared among 65 protein kinases (Hanks *et al.*, 1988). Nps1 contains six conserved sequence motifs identical or nearly identical to that of subdomains I, II, VI, VII, VIII and XI. In Figure 5, we compare the sequence of *NPS1* kinase-like domain with those of bovine cardiac muscle cAMP-dependent protein kinase and *S.cerevisiae CDC28* protein kinase. The kinase-like sequence motifs of Nps1, except for subdomain XI, exist in the region homologous to Snf2/Gam1. Although the sequence motifs of subdomains II, VII and VIII are also found in Snf2/Gam1, the sequence motifs of subdomains I, VI and XI are not.

Data base searches revealed that 492 bp sequence of *NPS1* downstream from *Bam*HI site is identical to *KDG1* 5′ non-coding sequence (Repetto and Tzagoloff, 1989). *NPS1* is physically mapped on chromosome IX by Southern blot analysis of *S.cerevisiae* A364A chromosomes separated by pulse-field gel electrophoresis (data not shown). Therefore, *NPS1* was concluded to be located on chromosome IX, 5′ flanked to *KGD1*.

NPS1 is essential

To examine the function of *NPS1*, a deletion allele of *NPS1* was constructed on a plasmid by replacing the 1.7 kb *Eco*RV−*Kpn*I sequence of *NPS1* with *URA3* gene and transformed *ura3/ura3* diploid RAY-3A-D (Figure 6A). The resulting uracil prototrophs were analysed for *NPS1* disruption by Southern blot analysis (Figure 6C). Four independently isolated heterozygous diploids were induced to sporulate and tetrads were dissected. In each tetrad, only two spores formed colonies and all of these colonies were uracil auxotrophs, indicating that *NPS1* is essential for spore viability (Figure 6B).

In order to examine whether *NPS1* and *SNF2/GAM1* share common biological functions, each gene was expressed in d416-1-4 (*MAT***a**, *gam1*Δ :: *URA3, leu2, ura3, STA1, inh*^{o}) and in YET100 (*MAT***a**/α *nps1*Δ :: *URA3/NPS1, leu2/leu2,*

ura3/ura3, his3/his3, trp1/trp1) on a high-copy number plasmid, YEp13. We tested the recovery of glucoamylase producibility of D416-1-4 transformants and the viability of haploid progenies yielded from YET100 transformants after sporulation. Each mutation could only be rescued by its own gene (data not shown). Thus, *NPS1* and *SNF2/GAM1* were concluded to have distinct functions.

NPS1 is a novel CDC gene

In order to characterize the phenotype of cells defective in *NPS1*, a conditionally expressive allele was constructed by introducing *GAL1* promoter sequence to the 5′ upstream region of *NPS1* in place of a 650 bp sequence from the *Eco*RI site (between −714 and −65) expected to contain the *NPS1* promoter. Haploid and diploid cells bearing the

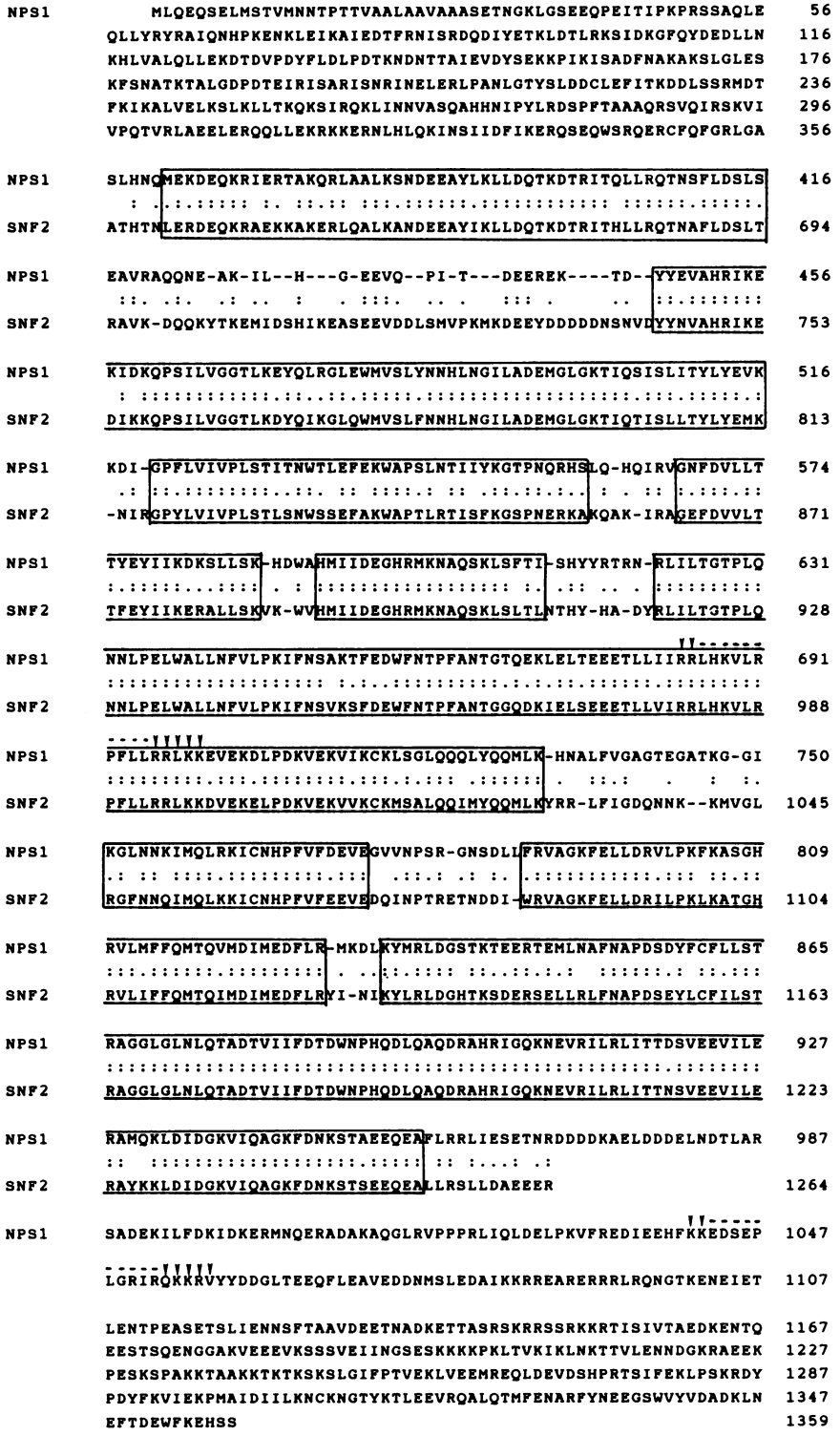

Fig. 4. Structural similarities between the *NPS1* and *SNF2/GAM1* proteins. The predicted amino acid sequence of *NPS1* was aligned with that of *SNF2/GAM1* with the maximum alignment program of DNASIS. Colons between amino acids designate identities, and dots represent conservative amino acid substitutions which are grouped as follows: A, G, P, S, T; D, E, N, Q; I, L, M, V; H, K, R; F, Y, W; and C. Sequences showing higher homology are boxed. Dashes indicate gaps for alignment. Amino acid sequence indicated with arrowheads and overlines are presumed dipartite nuclear targeting sequence reported by Robbins *et al.* (1991).

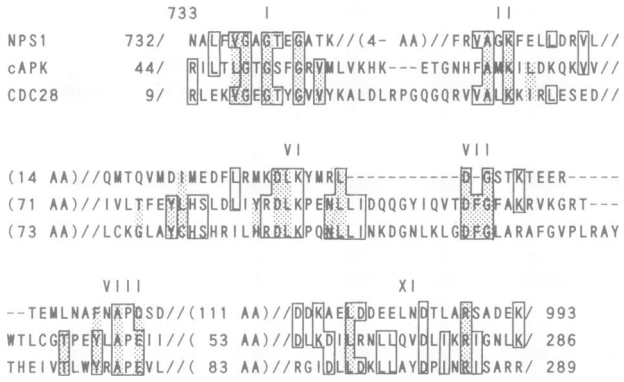

Fig. 5. *NPS1* amino acid sequence similarity with protein kinases. The predicted *NPS1* amino acid sequence was aligned with the catalytic domains of the bovine cardiac muscle cAMP-dependent protein kinase and *S.cerevisiae CDC28* protein kinase. Residues identical between *NPS1* and one or two of these protein kinases are boxed, and residues highly conserved in the protein kinases are indicated by shadowing. Dashes indicate gaps for optimal alignment.

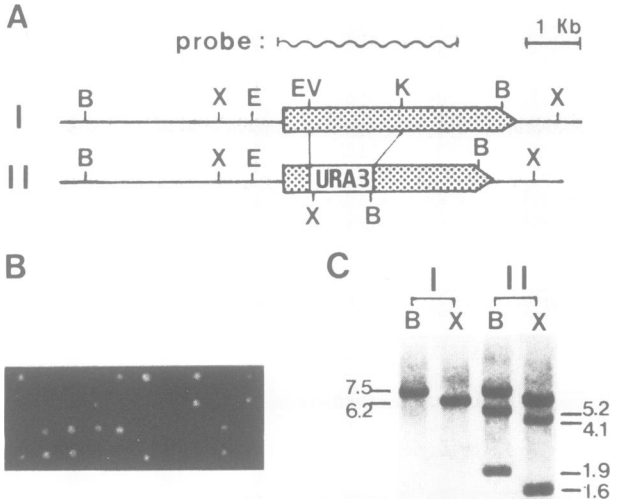

Fig. 6. Disruption of the *NPS1* gene. (**A**) Constructions of normal (I) and disrupted (II) *NPS1* allele. For restriction enzyme abbreviations see Figure 1. (**B**) Tetrad analysis of strain YET100. Haploid segregants from nine asci were dissected on YP glucose media and incubated at 30°C for 3 days. Spores from each ascus were lined in vertical rows. (**C**) Southern blot analysis. Genomic DNAs prepared from RAY-3A-D (wild-type) and YET100 were digested with *Bam*HI (lanes B) or with *Xba*I (lanes X). Hybridization was carried out with the 3.2 kb *Acc*I−*Stu*I fragment (panel A) of *NPS1* labeled with digoxygenin-labeled UTP.

*nps1*Δ :: *URA3* deletion allele and *pGAL1* :: *NPS1* can grow on YP galactose medium. As shown in Figure 7A, DD21 cells, one of the diploids bearing homozygous *nps1* null allele and *pGAL1* :: *NPS1*, arrested growth upon shift to YP glucose medium after one or two rounds of cell division. Under a microscope, >85% of DD21 cells arrested with a dumbbell shape 12 h after a shift to glucose medium (Figure 7B). The arrested cells were larger than galactose-grown cells (Figure 8A and B). DNA staining of the arrested cells with DAPI revealed that >90% of the dumbbell shaped cells have a single nucleus (Figure 8C). Location of the nucleus in arrested cells was heterogeneous: in ~80% of the arrested cells, the nucleus was located at or near the isthmus and in the remainder, the nucleus was located in the bud or in the mother cell. The terminal arrest morphology of Nps1-depleted cells was similar to that of temperature sensitive *cdc* mutants having defects in S, G_2 or M phases at their restrictive temperatures.

Figure 8D shows indirect immunofluorescence staining of microtubules in the arrested cells with anti-tubulin mouse monoclonal antibody and rhodamine-labeled antimouse IgG. The arrested cells possessed short microtubules that crossed the nucleus, suggesting that the separation of spindle pole bodies is completed but no elongation of pole-to-pole spindles occurs in these cells.

To characterize the terminal arrest phenotype further, we carried out flow cytometric analysis of DD21 cells stained with propidium iodide, and the results are shown in Figure 7C. At time 0 of incubation, cells were in various phases of the cell cycle. After 6 h of incubation in YP glucose medium, the number of cells with a G_2/M content of DNA (4n) clearly increased at the expense of all other cell cycle subpopulations, suggesting the requirement of Nps1 in G_2 or in M phase. The most striking feature of the Nps1-depleted cells was the appearance and increase of cells with an 8n DNA content after 9 and 12 h incubation in YP glucose medium. Although the percentage of dumbbell shaped cells with two nuclei measured under a fluorescent microscope after DAPI staining was <10% throughout the incubation in YP glucose medium (Figure 7B), the percentage of cells with an 8n DNA content in the 12 h population reached 47%. Moreover, the cells with an 8n DNA content also increased after 9 h incubation in YP glucose medium in the presence of 40 μg/ml of methyl benzimidazol-2-yl-carbamate (MBC), an antimicrotubule agent, which was added at 6 h of incubation (Figure 7D, c). The addition of MBC to DD21 cells in YP galactose medium resulted in the accumulation of cells in the G_2/M stage after 3 h incubation and no cells with an 8n DNA content appeared in a further 6 h incubation (Figure 7D, b). These results suggested that replication of DNA occurred in some of the Nps1-depleted cells without passage through mitosis. However, it was also possible that cells with higher DNA content (>4n) were generated as a result of mitochondrial DNA synthesis that continued even after the cessation of chromosomal DNA synthesis. To eliminate the effect of mitochondrial DNA synthesis, we isolated a ρ^- strain from DD21 by ethidium bromide treatment, and flow cytometric analysis was carried out with this strain. As the growth rate of DD21 ρ^- cells was 60% of that of ρ^- cells, the cells with an 8n DNA content appeared after 16 h of incubation in YP glucose medium, and increased to 30% after a further 8 h incubation (data not shown).

To confirm that re-replication of DNA occurs in an undivided nucleus, we next measured the fluorescence intensity within the nuclear region of Nps1-depleted DD21 ρ^- cells, which have a dumbbell shape and single nucleus, after DAPI staining by using a fluorescence microscopic photometer, ARGUS-100 photonic microscopic system. As shown in Figure 9, the nucleus in unbudded and large-budded cells grown in YP galactose medium showed fluorescence intensities (arbitrary units) between 5×10^3 and 9×10^3, and 10×10^3 and 19×10^3, with average values of 8×10^3 and 15×10^3 respectively. On the other hand, in the dumbbell shaped cells with a single nucleus that appeared after 24 h incubation in YP glucose medium, a nuclear fluorescence intensity of $>30 \times 10^3$ was frequently

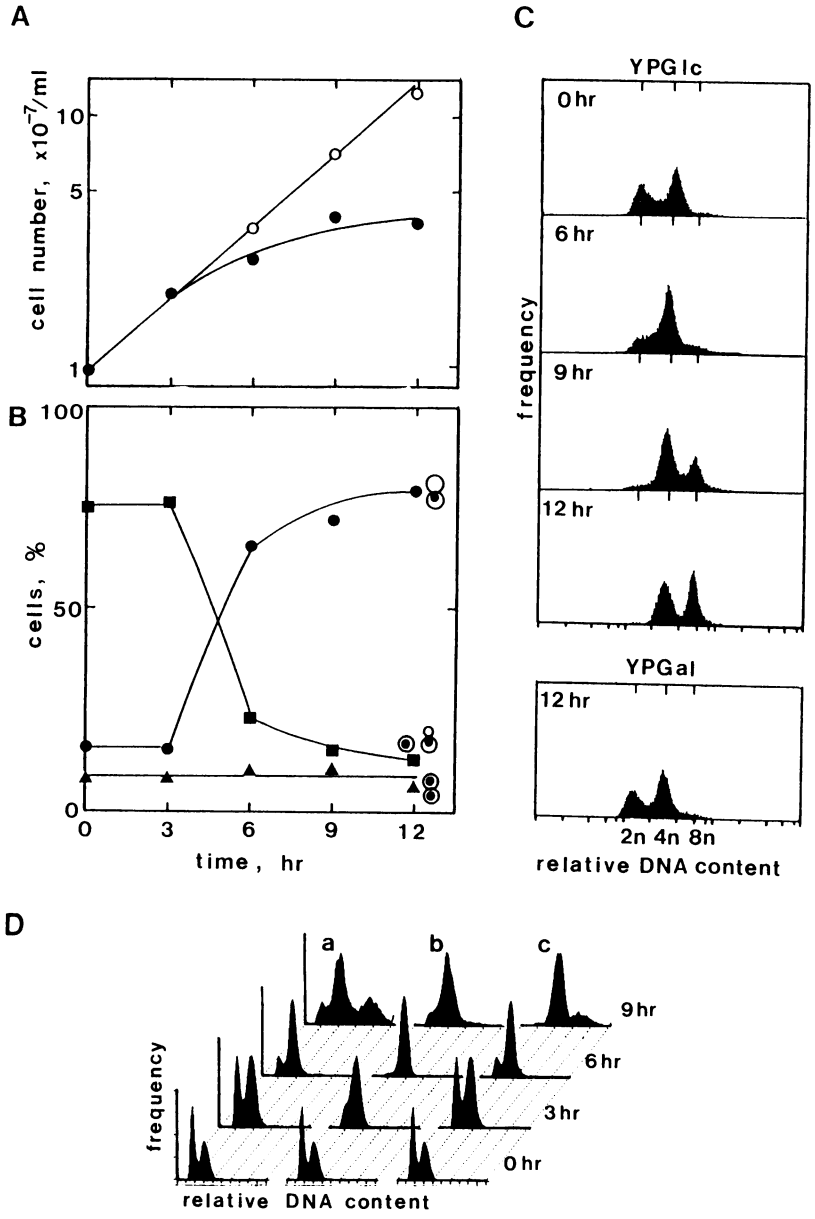

Fig. 7. Effects of Nps1 depletion on the growth and DNA content of the cells carrying galactose-inducible *NPS1* allele. (**A**) Growth of DD21 (*nps1*Δ :: *URA3*/*nps1*Δ :: *URA3*, *pGAL1* :: *NPS1*) cells in YP galactose (○) or in YP glucose (●) medium. Abscissa and ordinate are time and cell number respectively. (**B**) Analysis of cellular and nuclear morphologies of DD21 cells grown in YP glucose medium. Nuclear morphology was monitored by DAPI staining. (■), sum of unbudded and small-budded cells with single nucleus; (●), large-budded cells with single nucleus; (▲), large-budded cells with two nuclei. (**C**) Flow cytometric analysis of DD21 cells grown in YP glucose or YP galactose medium. Cultures analyzed in (A) were stained with propidium iodide and analyzed for DNA content using a flow cytometer. Results are plotted as the relative amount of DNA per cell versus the number of cells. (**D**) Flow cytometric analysis of DD21 cells grown in YP glucose medium in the presence of methyl benzimidazol-2-yl-carbamate (MBC). **a**, DD21 cells grown YP glucose medium without the addition of MBC; **b**, cells grown in YP galactose medium with the addition of 40 μg/ml of MBC at 0 h of incubation; **c**, cells grown in YP glucose medium and 40 μg/ml of MBC added at 6 h of incubation. The cells were harvested at the times indicated in the figure, stained with propidium iodide and analyzed for DNA content using flow cytometry.

observed. The results showed good agreement with that of flow cytometric analysis.

cdc29 maps to chromosome IX (Hartwell, 1974). Complementation of *nps1*Δ :: *URA3* by a temperature sensitive allele of *CDC29* at the restrictive temperature indicated that *CDC29* is not allelic to *NPS1* (data not shown).

NPS1 product is a nuclear protein

In order to detect the product of *NPS1*, an antibody against a quarter of the N-terminal segment of Nps1, between *Pst*I at position 80 and *Hin*dIII at position 1171, was generated by using a purified LacZ−Nps1 fusion protein produced in *Escherichia coli*. Yeast strains transformed with *pGAL1* :: *NPS1* or its vector YCpOS31 were grown in YP galactose medium, disrupted by vortexing with glass beads and fractionated into soluble and insoluble fractions by centrifugation. Western blot analysis was carried out on these protein preparations and the results are shown in Figure 10. Anti-Nps1 antiserum but neither anti-LacZ nor preimmune serum recognized a polypeptide with an apparent mol. wt of 160 kDa in the insoluble material (Figure 10A, data for preimmune serum are not shown). An increase in the density

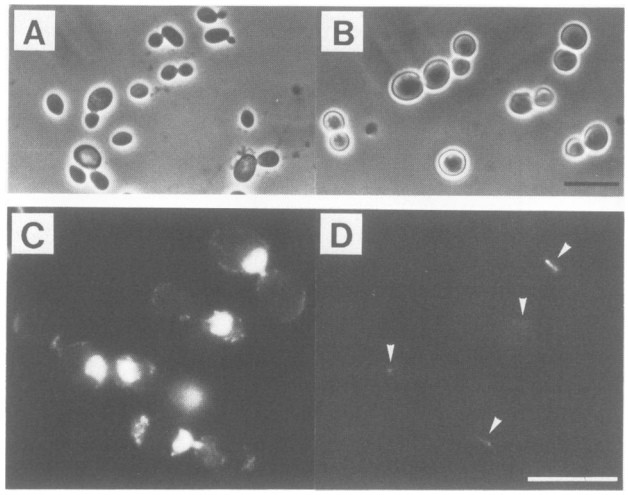

Fig. 8. Microscopic analysis of *NPS1*-depleted cells. DD21 (*nps1*Δ :: *URA3*/*nps1*Δ :: *URA3*, *pGAL1* :: *NPS1*) cells were grown in YP galactose (**A**) or in YP glucose medium (**B**) for 12 h and photographed under a phase-contrast microscope. The cells prepared in (B) were fixed and stained with anti-tubulin antibody and DAPI to show spindle (**D**) and nuclear (**C**) morphologies. Bar, 10 μm.

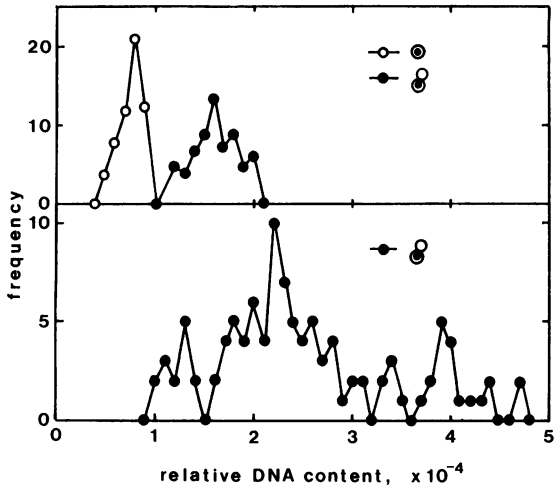

Fig. 9. Frequency distribution of the relative DNA content per nucleus stained with DAPI. DD21 ϱ⁻ cells were grown in YP galactose (upper panel) or in YP glucose medium (lower panel) for 24 h and stained with DAPI. The relative DNA content per nucleus in unbudded (○) and in large-budded cells (●) were analyzed by using a fluorescence microscopic photometer, and the results plotted versus the number of cells.

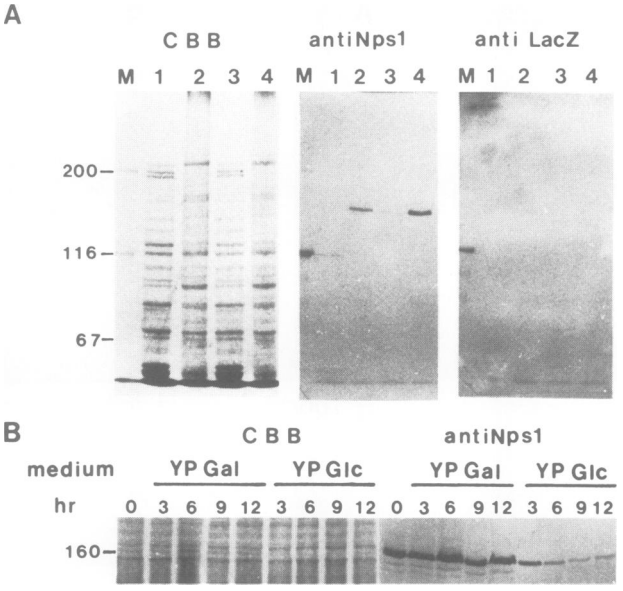

Fig. 10. Western blot analysis of *NPS1* product. (**A**) Immunological detection of *NPS1* product in wild-type (YET-H1) and in *NPS1*-overexpressing cells (YET-H1). YET-H0 and YET-H1 cells were grown to mid-log phase in YP galactose medium, and soluble and insoluble materials were prepared as described in Materials and methods. Proteins in soluble (lanes 1 and 3) or in insoluble (lanes 2 and 4) material were separated by SDS−PAGE, and stained with Coomassie brilliant blue (CBB) or with antibodies indicated in the figure after the transfer onto PVDF membrane. Samples from YET-H0, and from YET-H1 were run in lanes 1 and 2, and in lanes 3 and 4 respectively. Markers shown in kDa on the left were rabbit muscle myosin, *E.coli* β-galactosidase and bovine serum albumin. (**B**) Change of the immunoresponsive 160 kDa polypeptide in cells carrying the galactose-inducible *NPS1* allele. DD21 cells were grown in either YP galactose or YP glucose medium and harvested from each culture at different time intervals. Proteins in the insoluble material prepared from harvested cells were analyzed by Western blotting after separation with SDS−gels. Staining patterns with Coomassie brilliant blue (CBB) and anti-Nps1 antibody are shown on the left and right parts of the figure. Numbers at the top refer to the time in hours.

of the 160 kDa band was observed in the preparation from the cells carrying *pGAL1* :: *NPS1*. Moreover, in the cells carrying *nps1* null allele and *pGAL1* :: *NPS1*, a notable decrease of the 160 kDa band occurred upon shift to YP glucose medium (Figure 10B). The size of the immunoresponsive polypeptide showed good agreement with the size calculated from the deduced amino acids sequence of *NPS1*. The results indicated that the 160 kDa protein corresponds to the product of *NPS1*.

To determine the subcellular localization of the *NPS1* product, indirect immunofluorescence staining was carried out on wild-type and *NPS1*-overexpressing cells with affinity-purified anti-Nps1 antibody. Under a fluorescence microscope, faint staining of the nucleus in wild-type cells (data not shown), and bright nuclear staining with the cells overproducing Nps1 from the *GAL1* promoter was observed (Figure 11A). Comparison with the DNA staining pattern with DAPI (Figure 11B) indicates the presence of *NPS1* product in the nucleus. Neither preimmune mouse serum nor purified anti-LacZ antibody showed any fluorescent staining on both wild-type and Nps1-overproducing cells (data not shown).

Robbins *et al.* reported that the nuclear targeting sequence of nucleoplasmin is composed of two clusters of basic amino acids separated by 10 intervening amino acids, and this bipartite sequence motif is present in a number of nuclear proteins (Robbins *et al.*, 1991). Sequences that match to this motif are found in *NPS1* protein at positions 684 and 1041 (Figure 4).

NPS1 product may possess protein kinase activity

As described in the previous section, Nps1 contains sequence that is homologous to the catalytic domain of protein kinases. Among 11 major conserved subdomains defined by Hanks *et al.* (1988), a glycine-rich sequence in subdomain I (Gly-Xaa-Gly-Xaa-Xaa-Gly) and a Lys residue in subdomain II are proposed to form an ATP-binding site (Taylor *et al.*, 1990). The Lys residue in particular, is considered to be directly involved in the phosphotransfer reaction. The

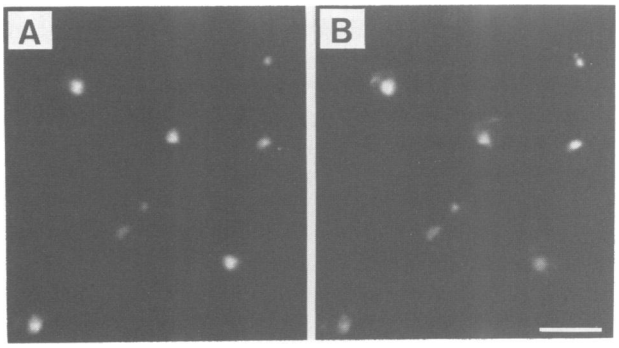

Fig. 11. Immunofluorescence staining of yeast cells. YET-H1 cells were grown to mid-log phase in YP galactose medium and stained with affinity-purified anti-Nps1 antibody and DAPI to show the localization of *NPS1* product (**A**) and nuclear morphology (**B**). Bar, 10 μm.

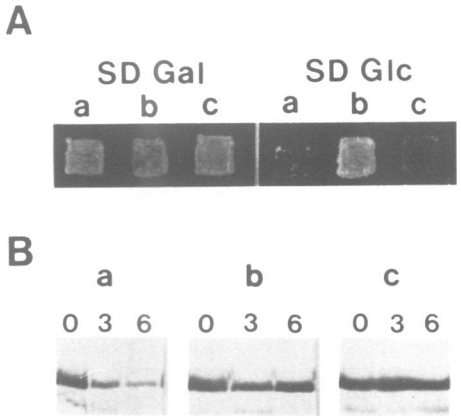

Fig. 12. Western blot analysis of *NPS1* and *nps1K792E* products. (**A**) Growth of YET101-15 (*nps1*Δ :: *URA3*, *pGAL1* :: *NPS1*) cells transformed with YEpHB3 (vector, **a**), YEp*NPS1* (**b**) and YEp*nps1K792E* (**c**) on SD galactose and on SD glucose plates after the incubation at 30°C for 5 days. (**B**) Western blot analysis on protein preparations from YET101-15 cells transformed with YEpHB3 (**a**), YEp*NPS1* (**b**) and YEp*nps1K792E* (**c**). Each strain was first grown in SD galactose medium and then shifted to growth in SD glucose medium. Cells were harvested at 0, 3 and 6 h incubation, and insoluble materials were prepared. Proteins in each preparation were analyzed by Western blotting with anti-Nps1 antibody after separation through SDS−polyacrylamide gels.

importance of this Lys residue is defined by biochemical and genetic studies in many protein kinases. This invariant Lys residue in Nps1 is expected to be Lys792. To examine the functional significance of Lys792, we used the method of site-directed mutagenesis to replace Lys792 of Nps1 by a Glu residue. The mutant gene (designated as *nps1K792E*) was cloned in YCpOS31 and the resulting plasmid was introduced into YET100. We dissected 60 tetrads formed in Leu^+ transformants of YET100 after sporulation. One and two viable spores were yielded from 11 and 49 tetrads respectively. All of these viable spores were uracil auxotrophs. This result suggested that Lys792 is essential for the function of the *NPS1* product. However, it is possible that *nps1K792E* was not expressed normally in yeast cells because of some additional mutation introduced during the process of plasmid construction. To detect the *nps1K792E* product, we transformed a haploid strain carrying *nps1* null allele and *pGAL1* :: *NPS1* (YET101-15) with a high-copy number plasmid, YEpBH3 (pBluescript, *ARS1, HIS3*), containing *nps1K792E* or *NPS1* (designated as YEp*nps1K792E* and YEp*NPS1* respectively). These transformants were grown in minimal selection medium containing galactose (SD galactose medium) followed by a shift to SD glucose medium. At various time intervals, cells were harvested by centrifugation and insoluble materials were prepared. The result of Western blot analysis of these protein preparations with anti-Nps1 antiserum is shown in Figure 12B. The 160 kDa polypeptide recognized by anti-Nps1 antiserum was present in all of the time 0 samples from YEpBH3, YEp*NPS1* and YEp*nps1K792E* transformants. The amount of 160 kDa polypeptide present in the latter two transformants did not decrease after 3 and 6 h incubation in SD glucose medium, while, it decreased notably in control vector transformants under the same conditions. As shown in Figure 12A, only the YEp*NPS1* transformant of YET101-15 could grow on SD glucose medium, but both of YEp*nps1K792E* and vector transformants could not. The data suggested that *nps1K792E* was translated into a protein product of the expected mol. wt. The results obtained here indicated that Lys792 of Nps1 is indispensable for its function.

Discussion

A new CDC gene, *NPS1*, which encodes a 160 kDa nuclear protein of *S.cerevisiae* was cloned and characterized. From the nucleotide sequence of *NPS1*, the gene was found to have striking homology to *SNF2/GAM1* which encodes a nuclear protein required for the transcriptional activation of multiple genes (Laurent *et al.*, 1991; Yoshimoto *et al.*, 1991). Recently, Tamkun *et al.* (1992) reported that the *brahma* (*brm*) gene, a regulator of *Drosophila* homeotic genes, also has structural similarity to *SNF2/GAM1*. The regions of similarity of these three proteins are found in residues between 447 and 923 of Nps1, 744 and 1219 of Snf2/Gam1 and 751 and 1230 of Brm, suggesting that this region may have a common function in these three proteins. However, the growth defect caused by *NPS1* disruption could not be rescued by the overexpression of the *SNF2/GAM1* gene and vice versa. Sequence homologies between Brm and Snf2/Gam1 also exist in the N- and C-termini of each protein in addition to the long homologous regions described above. On the other hand, both N- and C-terminal sequences of Nps1 are distinct from Snf2/Gam1 or from Brm. Further investigation is required for elucidating the relationships between structure and function(s) of each gene product.

Nps1 contains sequence motifs homologous to the catalytic domains of protein kinases. The substitution of Lys792 for Glu resulted in complete inactivation of Nps1. Therefore, *NPS1* product was suggested to have protein kinase activity. However, there are several significant differences between the kinase-conserved motifs defined by Hanks *et al.* (1988) and that in the *NPS1* kinase-like domain. The differences are, e.g. (i) an absence of sequence motifs that correspond to those of subdomains III, IV, V, IX and X, (ii) substitution of the conserved Asn residue in subdomain VI with an Arg residue, and (iii) the absence of Phe residue in subdomain VII. These differences raise a question for the nature of the presumed activity of this domain of Nps1. Considering the importance of Lys792 in Nps1, these differences may suggest some other enzymatic properties of this domain such as the capacity to phosphorylate amino acids other than serine, threonine or tyrosine, or the ability to

phosphorylate substrates other than proteins. Amino acid sequence surrounding Lys792 of Nps1, residues between 789 and 799, is identical to Snf2/Gam1 residues between 1084 and 1094. In addition, residues between 853 and 858 of Nps1 containing subdomain VIII sequence motif also show homology to the Snf2/Gam1 sequence between 1149 and 1154 (Figure 4). On the other hand, sequence motifs of subdomain I, VI and XI are not found in Snf2/Gam1. The former two subdomains are known to play an essential role in protein kinase catalytic domain (Hanks *et al.*, 1988; Taylor *et al.*, 1990). These sequence differences between Nps1 and Snf2/Gam1 in addition to the unique sequences in the N- and C-terminal domains of each protein may be responsible for the functional difference of the two proteins.

NPS1 was independently cloned by virtue of its homology to *SNF2* (*STH1*, Laurent *et al.*, 1992). While the amino acid sequence of *STH1* lacks seven amino acids between the Glu residue at 1073 and the Leu residue at 1079 of *NPS1*, the rest of the *NPS1* sequence is identical to that of *STH1*. Laurent *et al.* (1992) reported that *NPS1/STH1* contains amino acid sequence corresponding to conserved motifs found in two super families of >25 DNA and RNA helicases. Sequences resembling the consensus helicase motifs I to IV are found in the N-terminal part of the first consensus motif of kinase (between residues 492 and 703) and the consensus helicase motifs V and VI are found between that of kinase VIII and XI (between residues 856 and 910). These consensus helicase motifs exist within the region of homology shared between Nps1/Sth1, Snf2 and Brm. Putative helicase activity in this region offers a good basis to investigate common biochemical activity shared by these three proteins. However, invariant Lys792 is not included in these consensus helicase motifs. The complete loss of *NPS1* function by the substitution of Lys792 for Glu may not be due to the loss of helicase activity. Nps1/Sth1 may be a unique multifunctional protein carrying helicase and phosphotransferase activity. Further investigations are required to elucidate the biochemical functions of *NPS1* product. Analysis of the biochemical activities of the *NPS1* product is in progress in our laboratory.

NPS1 is essential for mitotic growth. The results of our analysis described here indicated that the gene is required for G_2 phase control. When diploid cells carrying the homozygous *nps1* null allele and the galactose-inducible gene on a plasmid (DD21) were incubated in YP glucose medium for more than three generation periods, re-replication of DNA occurred in some of the G_2-arrested cells (20–40%). The cells with an 8n DNA content also increased in the presence of MBC. MBC is known to bind tubulin and inhibit the polymerization of microtubules in many fungal cells including *S.cerevisiae* (Laclette *et al.*, 1980). The percentages of 8n cells in the absence and in the presence of MBC were 21 and 30% respectively. The decrease of 8n cells in the presence of MBC may be explained by the existence of the cells before G_2 arrest at the time of the addition of MBC (6 h incubation in YP glucose medium), because complete cessation of cell growth was observed only after 9 h in glucose medium as shown in Figure 7A. Our results suggest the possibility that some of the Nps1-depleted cells re-replicated DNA after arrest at the G_2 phase without passage through mitosis. In all eukaryotic cells, chromosomal DNA replication is strictly controlled to occur only once in the cell cycle preceding mitosis. Little is known about the mechanisms that prevent re-replication of DNA during the cell division cycle. In the fission yeast *Schizosaccharomyces pombe*, Broek *et al.* (1991) reported that several temperature sensitive mutants of $cdc2^+$ diploidized by transient heat treatment re-replicated DNA on recovery at the permissive temperature. They suggested that the memory of being in G_2 was lost in these $cdc2^{ts}$ mutants by the disappearance of $cdc2^+$ product, a protein kinase, which is assumed to be the factor responsible for such a 'G_2 memory'. In the recent analysis on the effects of several protein kinase inhibitors on cell cycle regulation, Usui *et al.* (1991) reported that the staurosporine analog K252a caused DNA re-replication in rat fibroblasts without an intervening mitosis to produce cells of higher ploidy. Because K252a selectively blocked the G_2 progression through to the M phase without inhibiting the 'start' events for S phase, they suggested that the drug may inhibit some G_2-specific protein kinase(s) distinct from cdc2 kinase which is required in both the G_1 and G_2 phases. Moreover, they have found that K252a also induces polyploidization in *S.cerevisiae* (M.Yoshida and T.Beppu, personal communication). Their finding suggests the presence of protein kinases responsible for 'G_2 memory' in *S.cerevisiae*. It is probable that Nps1 plays a role in the establishment of 'G_2 memory'. However, in some temperature sensitive mutants of *S.cerevisiae*, such as *cdc31* and *esp1*, cells with an increased ploidy are reported to arise under the restrictive conditions as a result of a defective mitosis (Schild *et al.*, 1981; Baum *et al.*, 1988). The possibility still remains that some of the Nps1-depleted cells leaked into a defective mitosis after arrest in the G_2 phase and progressed through the subsequent G_1 and S phases. In our analysis, we used a strain carrying a conditionally expressive allele of *NPS1*. Therefore, cell cycle arrest could not be induced in all of the cells at the same time. In the experiment shown in Figure 7D, the cells which had completed a defective mitosis might be included at the time of the addition of MBC, if such a defective mitosis occurred in Nps1-depleted cells at or shortly after the cessation of cell cycle progression. In order to clarify this point further, we are now constructing a temperature sensitive allele of *NPS1*.

Materials and methods

Yeast strains, media and genetic methods

Strains of *S.cerevisiae* used are listed in Table I. Yeast cells were grown in YP (1% yeast extract, 2% peptone) supplemented with 2% glucose or with 2% galactose plus 0.1% sucrose, as required. Synthetic minimal medium (SD, Sherman *et al.*, 1983) supplemented with the appropriate nutrient was employed to select for plasmid maintenance. Isolation of ϱ^- cells from DD21 was done by the method described by Slonimski *et al.* (1968). Standard genetic methods were as described (Ito *et al.*, 1983; Sherman *et al.*, 1983).

Sequence analysis

Several overlapping DNA fragments from *NPS1* were subcloned into M13mp18 or M13mp19 and sequenced by the method of Sanger *et al.* (1977), using a 7-deaza sequencing kit (Takara Shuzo Co., Ltd, Kyoto). The putative ORF was compared for sequence similarity to other proteins by DNASIS (Hitachi Software Engineering Co. Ltd, Yokohama) of various DNA and protein data bases.

Northern and Southern blot analysis

Total RNA was extracted from A364A cells grown to early-log (1×10^7 cells/ml), mid-log (3×10^7 cells/ml) or stationary phase (1×10^8 cells/ml) by the glass-beads method (Elion and Warner, 1984), separated on a 1% agarose gel, transferred to nylon membrane and subjected to Northern blot analysis (Sambrook *et al.*, 1989). The *NPS1* probe was generated by random-primed labeling of the *Eco*RV–*Kpn*I fragment of *NPS1* with [α-^{32}P]dCTP

Table I. Yeast strains

Strain	Genotype	Source
A364A	**a**, *ade1, ade2, ura1, his7, lys2, tyr1, gal1*	YGSC[a]
RAY-3A-D	**a**/α, *leu2/leu2, ura3/ura3, his3/his3, trp1/trp1*	K.Tanaka[b]
d416-1-4	**a**, *gam1*Δ :: *URA3, leu2, ura3, STA1, inh*°	H.Yoshimoto[c]
17048	**a**, *cdc29-1, ade1, ade2, ade7, ura1, his7, lys2, tyr1, gal1*	YGSC
YET-H0	**a**, *leu2, ura3, his3, trp1* (YCpOS31)	this study
YET-H1	**a**, *leu2, ura3, his3, trp1* (pGAL1 :: NPS1)	this study
YET100	**a**/α, *nps1*Δ :: *URA3/NPS1, leu2/leu2, ura3/ura3, his3/his3, trp1/trp1*	this study
YET101-15	α, *nps1*Δ :: *URA3, leu2, ura3, his3, trp1*, (pGAL1 :: NPS1)	this study
DD21	**a**/α *nps1*Δ :: *URA3/nps1*Δ :: *URA3 LEU2/leu2, URA3/ura3, HIS3/his3, trp1/trp1, his7/HIS7*, (p*GAL1* :: *NPS1*)	this study

[a]Yeast genetic stock center, Berkeley, CA.
[b]K.Tanaka *et al.*, 1989.
[c]H.Yoshimoto *et al.*, 1991.

(ICN Biomedicals Inc., Costa Mesa) and a Multiprime DNA labeling kit (Amersham).

Yeast genomic DNA was extracted from yeast strains by the method of Winston *et al.* (1983). The 3.2 kb *Acc*I−*Stu*I fragment of *NPS1* was labeled by random-primed incorporation of digoxygenin-labeled dUTP and used as a probe for Southern blot analysis. Labeling of the probe and immunological detection of the probe with anti-digoxygenin Fab fragments conjugated to alkaline phosphatase were done according to the manufacturer's instructions (Boehringer Mannheim).

S1 nuclease mapping

The probe DNAs evenly labeled with [α-^{32}P]dCTP were prepared as follows. Single-stranded DNAs were prepared from M13mp18 carrying the 985 bp *Eco*RI−*Eco*RV (MpEE) or 1885 bp *Eco*RI−*Hin*dIII (MpEH) fragments of *NPS1* in *Sma*I−*Eco*RI or *Hin*dIII−*Eco*RI sites respectively. DNAs were synthesized by using the Klenow fragment of *E.coli* DNA polymersae I with the sequencing primer, [α-^{32}P]dCTP and single-stranded DNAs of MpEE and MpEH. Double-stranded DNA synthesized from MpEE was cut with *Bam*HI and *Eco*RI (probe 1), and the DNA from MpEH was cut with *Hin*dIII and *Eco*RV (probe 2), or with *Hin*dIII and *Pst*I (probe 3). All DNA fragments were purified by agarose gel electrophoresis. Total RNA was extracted from early-log phase cells as described above. Poly(A)$^+$ RNA was purified by oligo(dT) cellulose chromatography. RNA (15 μg) was hybridized at 47°C with the ^{32}P-labeled probe DNA as described (Sambrook *et al.*, 1989). The mixture was treated with S1 nuclease (600 U/ml) for 30 min at 25°C. DNA fragments protected from digestion were analyzed in a 6% urea−polyacrylamide gel or a sequencing gel electrophoresis.

NPS1 gene replacement and plasmids

A mutant allele of *NPS1* was constructed by the one-step gene disruption method of Rothstein (1983). The 1.7 kb *Eco*RV−*Kpn*I fragment of *NPS1* was replaced by the *Hin*dIII−*Kpn*I fragment of pUC18-URA3-8 which contains the 1.2 kb *URA3* fragment in the *Bam*HI site of pUC18. This construction was made on the vector pBR322 carrying the 4.5 kb *Eco*RI−*Bam*HI fragment of *NPS1*. The DNA fragment containing *nps1* :: *URA3* was generated by *Eco*RI−*Sac*I digestion and was used to transform a *ura3/ura3* diploid strain RAY-3A-D (Table I). The genome containing the *nps1* :: *URA3* mutant allele gained novel *Xba*I and *Bam*HI sites originated from pUC18 multi-cloning sites. Selected diploid uracil prototrophs were determined for *NPS1* disruption by Southern blot analysis. One such diploid transformant, heterozygous at the *NPS1* locus (*nps1*Δ :: *URA3/NPS1*) was designated YET100.

Plasmids YEp*GAM1* and YEp*NPS1* were constructed as follows. The 9.7 kb *Sma*I−*Hpa*I fragment containing *GAM1* and the 5.8 kb *Eco*RI−*Xba*I fragment containing *NPS1* were generated from pHY3 (YIp1*GAM1*, Yoshimoto *et al.*, 1991) and from pKUT5 (YIp1*NPS1*, Figure 1) respectively. Each DNA fragment was inserted to the *Sma*I site of YEp13. pHY3 was a generous gift from Dr H.Yoshimoto.

Plasmid p*GAL1* :: *NPS1* was constructed to express the *NPS1* gene under the control of the *GAL1* promoter. The *NPS1* sequence between −65 and −714 expected to contain the *NPS1* promoter was deleted by exonuclease III digestion and replaced with the 0.8 kb DNA fragment containing the *GAL1* promoter enhancer sequence (Johnston and Davis, 1984). This construction was made on a plasmid pBluscript KS$^+$ containing the 7 kb *Eco*RI fragment of *NPS1*. The constructed chimeric *NPS1* gene was excised by *Xba*I digestion and was inserted into a centromeric shuttle vector YCpOS31 (pBR322, *CEN3, LEU2, TRP1, ARS1*). The resulting plasmid, p*GAL1* :: *NPS1*, was introduced into strain YET100 by selection for leucine prototrophy. The resulting strain, YET101 was sporulated, and haploid spores carrying a chromosomal *nps1*Δ :: *URA3* locus and p*GAL1* :: *NPS1* were germinated on YP galactose medium.

DAPI staining

Cell staining with the DNA specific dye 4,6-diamino-2 phenylindole (DAPI) was carried out according to published procedures (Williamson and Fennell, 1975). Measurement of fluorescence intensity of the nucleus in DAPI-stained cells was done with a fluorescence microscopic photometer, ARGUS-100 photonic microscope system (Hamamatsu Photonics K.K., Hamamatsu, Japan) by isolating the area of the individual nucleus and calculating the total intensity within the area through an ARGUS-100 control program.

Flow cytometric analysis

DD21 cells were grown in YP galactose medium at 28°C to 5 × 10^7 cells/ml, harvested by centrifugation, washed twice with 50 mM Tris−HCl, pH 7.5 and resuspended in YP galactose or in YP glucose medium at a concentration of 1 × 10^7 cells/ml. Both cultures were incubated at 28°C with shaking. At various intervals, culture containing 2−3 × 10^6 cells was withdrawn and the cells were fixed in 1 ml 70% ethanol and stored overnight at −20°C. The fixed cells were washed with 0.2 M Tris−HCl, pH 7.5 and suspended in 1 ml of the same buffer. After a brief sonication (5 s), RNase A was added to the suspension at a concentration of 1 mg/ml, and the suspension was incubated at 30°C for 4 h. The RNase A-treated cells were washed with 0.2 M Tris−HCl, pH 7.5, suspended in 0.1 ml of propidium iodide (PI) solution containing 50 μg/ml PI, 0.1% sodium citrate, 10 mM NaCl and 0.19% Nonidet P-40, and incubated on ice for 15 min. The fluorescence intensities of stained cells were analyzed with an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Antibodies and immunostaining

A LacZ−NPS1 fusion protein that contains 116 kDa LacZ protein fused to 40 kDa of the N-terminal part of NPS1 protein was produced in *E.coli* pop2136 cells transformed with pEX2*NPS1*. The plasmid pEX2*NPS1* was constructed by inserting the 1.1 kb *Pst*I−*Hin*dIII fragment from *NPS1* (positions 80−1171) into pEX2 (Stanley and Luizio, 1984) between the *Pst*I and *Hin*dIII sites. The fusion protein was purified from SDS gels and used to immunize mice. We also prepared antiserum against *LacZ* product from the lysate of *E.coli* carrying pEX2, and used this as a control for the immunodetection of *NPS1* product. For the immunostaining of yeast cells, antisera were purified on an affinity column. Affinity gel was prepared by coupling a purified LacZ−NPS1 fusion protein to cyanogen bromide activated-Sepharose 6MB (Pharmacia-LKB Biotechnology AB, Uppsala). Protein preparations were run on a 6% SDS−polyacrylamide gel, transferred to PVDF membrane (Millipore) and subjected to immunoblotting as described (Towbin *et al.*, 1979). Immunostaining of yeast cells was carried out by the method of Kilmartin and Adams (1984). Horseradish peroxidase-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-mouse IgG F(ab') fragment and anti-β-tubulin mouse monoclonal antibodies were obtained from Cappel Organon Tekinika Corporation (West Chester, PA), Immunotech S.A. (Marseilles, France) and Boehringer Mannheim respectively.

Oligonucleotide-mediated mutagenesis

To obtain the mutant *nps1K792E* gene which contains a codon for Glu in place of the codon for Lys792, the 700 bp *Kpn*I−*Sac*I fragment of *NPS1*

was cloned in M13mp18 and single-stranded template was prepared. The mutagenesis was carried out by using MUTAN-G kit (Takara Shuzo Co., Ltd, Kyoto) with a 25 bp oligonucleotide, AGAGTAGCGGCCGAGTT-TGAATTAC, and the single-stranded template according to the manufacturer's instructions. After confirmation of the mutation by M13 dideoxy sequencing, the DNA fragment containing the mutation was excised by *Kpn*I–*Hin*cII digestion. The wild-type sequence between *Kpn*I and *Hin*cII in *NPS1* was replaced with the mutant one on a plasmid pBluescript KS$^+$. The resulting mutant gene, *nps1K729E*, was excised by *Xba*I digestion and cloned into the *Xba*I site of YCpOS31.

Preparation of yeast proteins

Cells harvested from various cultures were washed twice with ice cold 20 mM *N*-2-hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid (HEPES)–KOH, pH 7.0 containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of leupeptin and pepstatin and 2 μg/ml each of antipine, chymostatin and aprotinin (buffer A) and resuspended in the same buffer and disrupted by vortexing with glass beads at 4°C. Clear supernatant obtained by centrifugation (30 000 *g*, 15 min) was used as the source of soluble proteins. The resulting precipitates were washed three times with buffer A and used as the source of insoluble protein. Protein concentrations were determined by the method of Bradford (1976).

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